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PRINCIPAL INVESTIGATOR: Nicolas G. Bazan, M.D., Ph.D.

CONTRACTING ORGANIZATION: Louisiana State University
School of Medicine
New Orleans, Louisiana 70111-2822

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Nicolas G. Bazan, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Louisiana State University
  School of Medicine
New Orleans, Louisiana 70111-2822

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FOREWORD

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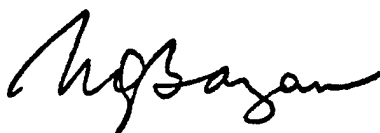
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The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Neurochemical Protection of the Brain, Neural Plasticity and Repair**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC#
Rat (Sprague-Dawley)	125	125	1045, 1046, 1482

Investigator Signature

TRAUMATIC BRAIN INJURY

Introduction

The development of chronic epilepsy is a very serious complication of head injury, neurodegenerative diseases, brain tumors, and exposure to neurotoxic agents. Head injury is often associated with loss of short-term memory, indicating trauma to the hippocampal formation, the brain region most commonly associated with epileptic brain damage. Underlying the formation of epilepsy (epileptogenesis) is proposed to be a vicious cycle initiated by the loss of neurons. In an attempt to repair and/or replace lost synaptic connections, the brain can develop aberrant synaptic circuits that permit the propagation and amplification of waves of excitatory neurotransmission, eventually resulting in prolonged or repeated seizures (status epilepticus). The massive amounts of excitatory amino acids released during these episodes can stimulate further neuronal loss (excitotoxic damage), the formation of more aberrant synaptic circuits, and further seizures.

It is now apparent, however, that even brief, sporadic seizures, such as those induced in the kindling model of epileptogenesis, may induce neuronal loss in vulnerable brain regions[1]. Kindling is a widely-used animal model of chronic epilepsy in which repetition of a given electrical stimulus to a discrete focal point in the limbic system of the brain gives rise to a fixed sequence of events. This sequence is characterized by a progression in both the duration of the electrographic seizures (afterdischarges), and in the severity of the accompanying behavioral seizures until a stable plateau is reached. Subsequently, the same stimulus, which at the beginning of the process elicited a minimal afterdischarge, will then elicit a protracted afterdischarge and stereotyped generalized convulsions characteristic of kindled seizures[2]. Kindled seizures can then subsequently be elicited in treated animals even when the stimulus is witheld for several months, which establishes that chronic alterations have occurred in the kindled brain analogous to those in the epileptic human brain. Hippocampal kindling has been shown to induce collateral sprouting of mossy fibers, and progressive loss of interneurons in the hilus of the dentate gyrus, and in the CA1[1,3].

Kindling is an experimental model of mesial temporal lobe epilepsy (MTLE), whereby repeated application of initiative subconvulsive electrical stimulation results in progressive motor seizures, culminating in generalized clonic motor seizures[3-5]. We believe that changes of certain gene expressions are required for kindling epileptogenesis and maintenance of kindling effect, and interference with expression of these genes will inhibit kindling epileptogenesis. In this project, we hypothesize that: 1) A single kindling stimulation induces acute gene expression. 2) Repetitive stimulation induces accumulation of acute response gene products, which in turn activate sustained neurotrophic gene expression and lead to synaptic reorganization, neuron death and proliferation. 3) Platelet activation factor (PAF) mediates stimulation of induced gene expression in kindling.

The Rapid Hippocampal Kindling Model of Traumatic Head Injury

Kindling was introduced by Goddard and his colleagues early in 1969[2]. It is defined as the phenomenon in which repeated administration of an initially subconvulsive electrical stimulus to animal brain results in progressive intensification of seizure activity, culminating in a generalized convulsion. Because behavior and physiology manifestation of limbic kindling resembles human mesial lateral temporal epilepsy (MLTE), kindling was accepted as an experimental model for temporal lobe epilepsy soon after its introduction[6,7].

Kindling can be achieved in a variety of brain regions[2], but amygdala and hippocampus are most frequently studied. Kindling from amygdala was found to be most efficient, this explains the reason why this model is most extensively used. In kindling from amygdala and many other regions, morphology and molecular biological alteration were found mainly in hippocampus. This included neuron loss[1,3,8-11], mossy fiber sprouting[8,10], modification of neurotransmitter receptors[13-16], and induction of gene expression[17-21], suggesting that hippocampus plays a very important role in kindling. Also hippocampus sclerosis is the main pathological founding in human MLTE. Surgical removal of hippocampus will reduce or eliminate epileptic seizure of MLTE patients, implying hippocampus is critical to seizure origin, and probably it is the main site of seizure origin[22]. In this project we use hippocampus kindling in order to simulate human epileptic seizure originated in hippocampus.

There are a variety of stimulation protocols to induce kindling in rats. The classical kindling uses tetanic stimuli with frequencies of 60 Hz and train duration of 1 second, delivered daily[2]. Using this stimulation protocol, hippocampus kindling requires as long as 50 to 77 days. Loathman[23] in 1985 developed a rapidly hippocampal kindling protocol. His protocol was based upon the administration of a 10 second 10, 25, 50 Hz train of stimulation every 5 minutes during a period of 6 hours. This protocol was found capable of inducing progressive seizure, but the seizure intensity was not increasing as smoothly as classical kindling. In 1993, he develops another improved rapid hippocampal kindling protocol[24]. This protocol differs from the former one on that the stimulation was given 12 times daily for a total of 4 days with an inter-stimulus interval of 30 minutes. Hippocampal kindling using this stimulation protocol progressed rather smoothly and smaller variations were observed between individual animals.

The Requirement of Gene Expression Regulation in Kindling

It has been clearly established that kindling can induce neuron loss and mossy fiber sprouting[1,3,8-11], but it is still controversial whether kindling epileptogenesis requires neuron loss[3,8,25,26] or mossy fiber sprouting[8,10,25]. It is well known that gene expression is required in neuronal apoptosis and neurite growth, so is almost certain that regulation of gene expression is involved in kindling-induced neuronal death and neurite reorganization. Gene expression induction is observed after seizure[17-21], and inhibition of protein synthesis suppresses kindling development[27], suggesting that gene expression is required in kindling development. It is now known that kindling induces both acute and long-term changes in gene expression. Acute gene expression upregulation includes immediate early genes, neurotrophic factors and other genes, which are temporally upregulated few minutes to hours after seizure and back to normal within one day. Immediate early genes such as c-fos[17,18,20,28], c-jun[18,28], jun-B[18], krox-24[18], NGFI-A (also known as zif-268)[29], erg-1[30] and tissue-plasminogen activator[31] are induced within hours after seizure. Neurotrophic factor genes such as BDNF, NGF, NT-3[32] and their receptor genes such as trk B and trk C[33] are acutely induced by seizure, while genes such as mineralocorticoid and glucocorticoid receptors[34] are also increased after a stimulation-induced seizure. Genes exhibit long term changes after kindling. This includes calcium calmodulin dependent protein kinase II (CaMK II)[35], NMDA receptor[36], kainate receptor[37], AMPA receptor[38], GABA$_A$ receptor[39,40,41], neuropeptide Y(NPY)[42], prodynorphin and proenkephalin[43,44,45], vasopressin[46,47],

and ligatin[48]. Most of the above genes do not sustain changes for longer than a few weeks. For those genes such as ligatin that have sustained change of expression, none has been shown to be involved in elevated neuronal excitability. Nevertheless, kindling induced hyper-excitability remains for a few months or, in some cases, for the rest of animals' life[2], suggesting that kindling is maintained probably not by elevation of certain gene expression, but by the formation of new neural circuits[49, 50], apoptosis of existing neurons[51, 52] and formation of new neurons[11,51]. All these process requires temporal upregulation of genes expression that involves apoptosis, neuroprotection, and neurite growth. This is in agreement with the fact that kindling involves transient gene expression regulation and inhibition of protein synthesis to block kindling development[21].

PAF is a Potential Mediator of Gene Expression in Kindling

Platelet-activating Factor (PAF) was originally identified in the 1970s as a substance released from activated basophils[53]. It was then detected in a variety of inflammatory and immune-related cells, such as leukocytes, monocytes, macrophages, endothelial cells and platelets[54]. It is a potent mediator in anaphylaxic and inflammatory responses[54], as well as an important substance in reproduction[56,57] and development[57,58]. In the late 1980s, PAF was also detected in mammalian brain[59] and shown to be significantly increased after seizure[60]. PAF is synthesized by neurons[61,62] and glial cells[62] in culture, but it is commonly accepted that PAF accumulates in brain tissue after stimulation, mainly originating from neurons[63]. PAF is implicated in gene induction, ion channel modification, and enzyme activation[64,65], which qualify it to be a potent mediator of gene expression induced by kindling stimulation.

In vitro, PAF is a powerful modulator of proteins and enzymes capable of mediating gene expression in neurons. PAF can activate NFκB[66], MAPK[67,68], and modulate NMDA receptor function[69,70]. NFκB is a potent activator of immediate early gene transcription[71]; MAPK is responsible for modulation of gene expression[72]; and, the NMDA receptor is a critical mediator of kindling development[73-81] and kindling-induced mossy fiber sprout[82,83]. PAF can also enhance glutamate release in LTP[84], increase intracellular calcium concentration[85], and promote apoptosis[86].

There are two known pathways of PAF synthesis in neurons[87]. One is the de novo pathway that utilizes CDPcholine and alkylacetylglycerol and is catalyzed by a specific

phosphocholinetransferase (PAF-PCT). The other is the remodeling pathway ending with the reaction catalyzed by lyso-PAF acetyltransferase (lyso-PAF AcT), utilizing lyso-PAF, a product of phospholipase A2 activity, and acetyl-CoA. It has been established that the remodeling pathway is activated in hippocampus during ischemia[88]. Cytosolic phospholipase A_2 is activated by calcium influx after glutamate treatment of neurons[89], supporting the idea that PAF synthesis is elevated during kindling through remodeling pathways.

PAF has at least two high-affinity binding sites in the brain[64, 90], one in synaptosomes, and the other in microsomes. They differ in their antagonists specificity. The synaptosomal site is selectively blocked by an experimental drug, BN52021, and inhibition of PAF binding to this site inhibits PAF mediation of hippocampal LTP. This site corresponds to the G-protein coupled PAF receptor cloned in mammalian or human lung[91], spleen[92], human leukocytes[93] and heart[94]. The microsomal site is blocked by BN50730, and this site is responsible for PAF induced immediate gene expression[95]. The identity of this binding site is still unknown.

COX-2 as a Possible Mediator of PAF-Induced Apoptosis in kindling

There are two isoforms of cyclooxygenases (COX) responsible for prostaglandin synthesis[96]. COX-1 is the constitutive form, while COX-2 is the inducible one. COX-2 is known as a very important mediator in inflammatory response[96] and an anti-apoptotic enzyme in cancer growth[97]. Recently, COX-2 was found to mediate neuronal death caused by brain ischemia[98], seizure[99] and chochicine toxicity[100]. Since PAF antagonists inhibit COX-2 gene transcription after seizure[101], it is postulated that COX-2 mediates PAF induction of neuronal apoptosis during kindling.

BODY
Previous work

In previous reports (years 1 & 2), we have shown that COX 2 mRNA and protein levels are up-regulated during cryogenically-induced vasogenic brain edema in rats. COX 2 protein levels peak at 4-5 hours post-injury, 2-3 hours after the peak in COX 2 mRNA levels. The induction is sustained up to 24 hours post-injury. A role for platelet-activating factor in COX 2 activation was implied by the ability of the specific intracellular PAF receptor antagonist BN 50730, and the novel PAF antagonist LAU 503, to block this induction at least partially. PAF

receptor antagonists and glucocorticoids (inhibitors of COX 2 up-regulation) also reduce blood-brain barrier breakdown in this model, implying a role for PAF-induced COX 2 activation in edema formation. We used electrophoretic mobility shift assays (EMSAs) to show that the DNA binding activities from rat brain nuclear extracts specific for AP2, CREB, GAS/ISRE and NFkB decreased during the cryogenic injury. We presented evidence of a novel DNA binding protein in rat brain nuclear protein extracts which binds to a region (-460 to -258) of the COX 2 promoter. This protein was present only in uninjured brains, and disappeared during cryogenic injury. We speculated that this protein represented a repressor of COX 2 expression, the removal of which during injury was an important step in the increase of COX 2 transcriptional activity.

In years 2 and 3, the rapid kindling model was used to assess the effects of the PAF antagonist BN 50730 on the development of electrographic and behavioral seizures. Drug-treated animals showed a statistically-significant lower increase in evoked after-discharge duration (ADD), and a slower development of behavioral seizures. Interestingly, ADDs were not significantly different between drug-treated and control groups after the first day, but diverged later during the process. This indicated that BN 50730 was not acting as an anticonvulsant, but was inhibiting some process underlying the development of kindled seizures. Preliminary histological studies suggested that BN 50730 was protective against cell loss in the hilus of the dentate gyrus. We also determined that, while some initial cell death occurs immediately within the acquisition phase, many nuclei survive throughout the development and retest phases of kindling development. The large drop in hippocampal, dentate gyral, and hilar cells occurred as a gradual process as a result of continual seizure episodes following the kindling process. We concluded that neural sprouting and rewiring, known to occur early in the kindling process, are either an independent process, separate from that producing apoptosis, or are a continual process that accompanies a slow, low level apoptotic process.

During year 4, we expanded our studies of transcription factor regulation during the brain edema model in the rat by identifying the factors, specifically induced by PAF, that regulate COX 2 promoter activity. This entailed using cell lines in which PAF induces COX 2 transcription. During this project, sequence data for the COX 2 promoter from other species (mouse and human) became available, and there were apparently interspecies variations in promoter structure. Therefore, if COX 2 gene activation were to be a potentially useful therapeutic target, finding it out whether the factors important in COX 2 promoter activation in

our rat model were similar to those activating the human promoter was necessary. For these studies, using for our EMSAs nuclear protein extracts from cell lines in which PAF induces COX 2 expression was necessary. We used a rat glioma/mouse neuroblastoma hybrid cell line (NG108-15), and a human B lymphoblast line (IM9) as our PAF-responsive cell lines.

We also wanted to characterize the putative rat COX 2 repressor protein further, and to learn if a homologous protein was involved in human COX 2 promoter activity. For this we used DNA-magnetic bead affinity technology, designed to isolate and purify proteins that bind to a specific DNA sequence. The ability of PAF to induce the injury-associated nucleoprotein identified in the brain edema model (Progress Report, year 2) was assessed in the cell lines. Nucleoprotein extracts were prepared from NG108-15 and IM9 cells treated for different times with PAF.

Intracerebroventricular injection of rats with BN 50730 before the stimulus inhibited the seizure-induced up-regulation of COX 2 expression in hippocampus, as well as the downregulation of transcription factor activities triggered by seizures, similar to our observations using the brain edema model (Progress Report, year 2). Treatment of IM9 cells with PAF induced a similar pattern of downregulation of the transcription factors AP-2, CRE and NFkB, which is sensitive to BN 50730. We concluded that the PAF response in the human cell line in vitro is qualitatively similar to that of the BN 50730-sensitive response to neurotrauma in the rat.

Objectives, Year 5

Plasticity modifications induced by traumatic head injury or seizures are not well understood. The objective of the current period of work was to use the kindling model of epilepsy to investigate COX-2 (cyclooxygenase-2) and $cPLA_2$ (cytosolic phospholipase A_2) upregulation of gene transcription. Figure 1 summarizes our hypothesis. Briefly, stimulation induces neuron depolarization and calcium influx, thereby activating $cPLA_2$. $cPLA_2$ catalyzes PAF and arachidonic acid (AA) synthesis. PAF further activates gene expression of COX-2 and $cPLA_2$. AA is a substrate of COX-2 that catalyzes prostaglandin synthesis, inducing neuronal apoptosis. The increased expression of COX-2 and $cPLA_2$ in neurons acts as positive feedback and, therefore, catalyzes the evident effect on gene expression and apoptosis (Fig 1A). Figure 1B

summaries our overall hypothesis that COX-2 gene expression induces apoptosis of inhibitory interneurons, whereby establishing an unchecked route of stimulation transmitted from the hippocampus to the motor cortex, resulting in seizure.

Material and Methods

Surgery

Wistar rats weighing 180-350g were anesthetized (ketamine 100 mg/kg, xylazine 10mg/kg, i. p.) and polyimide coated stainless steel bipolar electrodes were stereotactically implanted in the right ventral hippocampus (AP –3.2, ML –5.2, DV –6.5 to dura; according to Pellegrino's atlas published in 1979). The rats were allowed 2 to 4 weeks to recover from surgery.

Kindling

Figure 2A depicts the setup of the kindling model. Adult male Wistar rats were placed in a transparent cage through which we could observe seizure behavior. Figure 2B demonstrates the implantation of the electrode into rat hippocampus. Briefly, a Teflon coated tripolar electrode is implanted into the circled region in right ventral hippocampus. Two ends of the electrode are inserted into the hippocampus, serving the purpose of stimulation and recording, the third end of the electrode is attached to the outside surface of the skull by screws serving as ground. The electrode is firmly cemented to the skull using four small screws. A cable is then connected to the electrode implanted in the right ventral hippocampus of the rats. Kindling electrical stimulation is delivered and after-discharge inside the hippocampus monitored through the same electrode. Kindling stimuli are applied 12 times every other day for 9 Days, with 30 minutes intervals between stimulation (see Figure 3). 59-66 days after kindling initiation, COX-2, COX-1 and cPLA2 mRNA content in hippocampus, cortex, and cerebellum of both sides were measured 2 hours after stimulation using RT-PCR. The seizure behaviors are scored, using the method of Loathman. The ADD are also monitored.

Kindling Confirmation

Kindling was confirmed 55 days after the kindling initiation date, using one or two trains of the same stimulation used in kindling. The animal is considered kindled if a stage 5 seizure is elicited after stimulation. Seizure score is defined as described by Racine et al. (1972):

1. facial clonus, stand still, wet dog shaking
2. head nodding
3. fore limb clonus
4. rearing, loss of balance
5. falling

Sampling and RT-PCR

Four to eleven days after kindling confirmation, the rats were stimulated with one single train of stimulation, and cortex, hippocampus and cerebellum of both sides were sampled 2 hours after stimulation. Total RNA was extracted from the tissues, using RNA STAT-60 from Tel-Test Inc. RT-PCR was performed to determine the mRNA level of COX-2, $cPLA_2$ and COX-1. G6PDH was used as control.

RESULTS

During the kindling stimulation process, after 0.1 second of electrical stimulus, a recording of EEG electrical activity was produced for up to 12 minutes. At the end of the experiments, computer analysis was produced, and ADD events were compiled and analyzed for duration and frequency. ADD as a function of the number of stimuli is shown in Fig. 4A. The profile reflects a progression in the ADD as a result of repetitive stimulus. The events reached an average maximum duration of 150 sec, which correlated at the same stimulation period with class 4 – class 5 type seizures, as shown in Figure 4B. Seizure classifications were obtained, according to Racine (1972). Per day stimulation produces a progressive increase in seizure response and ADD, and reaches plateau by the end of the stimulation set , but the slope increases as animals approach the fifth stimulation set. After a two week recovery, a single simtulation generated a full scale seizure, confirming that the animal was kindled.

When the COX-2 message was studied in rat hippocampus and cortex of kindled and unkindled animals, receiving a single stimulation and sacrificed after two hours, there was a significant increase in both the right (stimulated site, $p< 0.01$) and left hippocampus ($p< 0.001$) (Fig 5A). However, in cortex, there was a significant increase only in the kindled group (Fig 5B) ($p< 0.01$ for both left and right cortex.) There was not change in COX-1 message in any of the animals studied (data not shown.) Study of $cPLA_2$ mRNA revealed the same pattern of change as that of COX-2 mRNA in hippocampus (Fig 6A), while not significant changes were obtainted in $cPLA_2$ mRNA in rat cortex (Fig 6B). Gene transcription of COX-2 and $cPLA_2$ had not significant changes in cerebellum after stimulation of either kindled or non-kindled rats. Changes in left and right sides of cortex and hippocampus revealed no significant differences, indicating changes were not associated with trauma induced by electrode implantation.

Kindled or non-kindled animals were subjected to a single kindling stimulation, then sacrificed in a time course up to 24 hours. The profile analysis for COX-2 mRNA in hippocampus and cortex of both groups are shown in Fig 7B. Hippocampal tissue shows no differences, with the exception of a pronounced peak after 1 hour stimulation. Such differences were not significant. However, a completely different profile is apparent in kindled and non-kindled cortex: kindled cortex shows a two step curve, the first of which peaks after 2 hours, the second at about 12 hours. Figure 7A is a representative agarose gel of COX-2 PCR products.

A time course of expression levels of cPLA$_2$ mRNA after a single stimulation in kindled rats reveals a differential profile for the hippocampus, as compared to cortex (Fig 8B). The slope of both respective curves run parallel up to four hours, with hippocampus reaching a plateau and remaining slightly over-expressed to 24 hours. Cortex shows a 200% increase by 12 hours, remaining elevated to 24 hours. A representative agarose gel of cPLA$_2$ PCR products is shown in Fig 8A.

Conclusions

1. Kindling stimulation triggers upregulation of both COX-2 and cPLA$_2$ genes in both the implanted and the contralateral hippocampus in kindled and non-kindled rats.

2. Only kindled animals show an upregulation of COX-2 expression in cortex. One kindling stimulation: a) in the left hippocampus, results in a sustained upregulation of COX-2 mRNA with no differences between kindled and non-kindled animals; b) in the left cortex, induces a biphasic increase in COX-2 mRNA only in kindled animals, with a sharp peak by 2hr, followed by a second peak at 12 hr and sustained to 24 hr.

3. Kindled and non-kindled animals show the same pattern of changes in cPLA$_2$ 2 hours after a single stimulation.

4. Time course of cPLA$_2$ mRNA levels after a single stimulation reveals a differential response between hippocampus and cortex, indicating a different latency to signal-response activity for these tissues.

5. Cortex shows a delayed enhancement of cPLA$_2$ by 12 hrs, which correlates with the second peak of expression for the COX-2 gene. The delayed over-expression of these two enzymes could be the result of intense neurologic activity. This may implicate the cortex as the final location of recurrent epileptogenesis.

These results may indicate that kindling triggers the establishment of new circuitry, which is involved in generating a significant response in both sides of cortex for COX-2 and cPLA$_2$.

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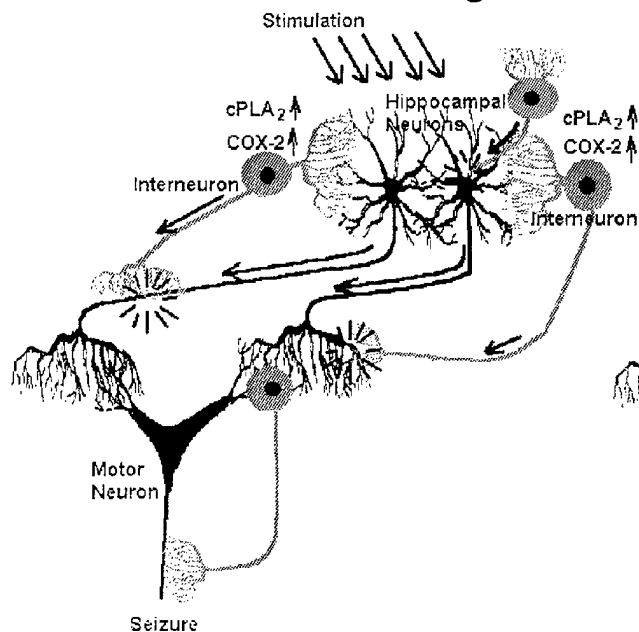
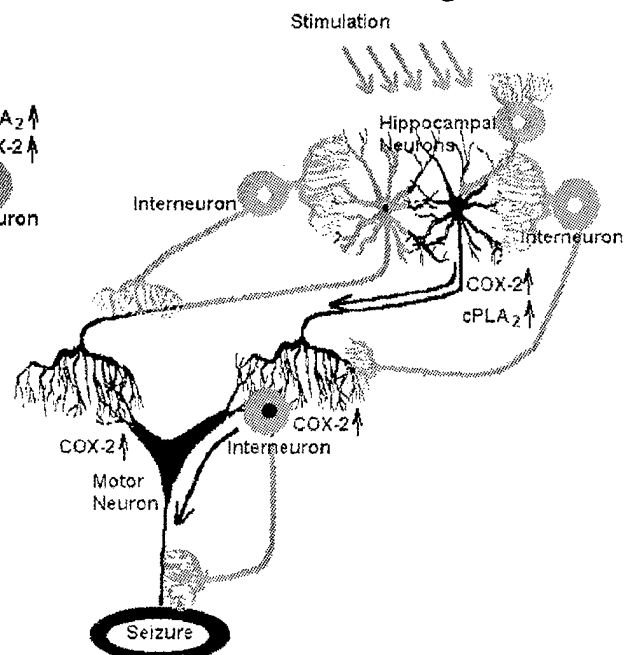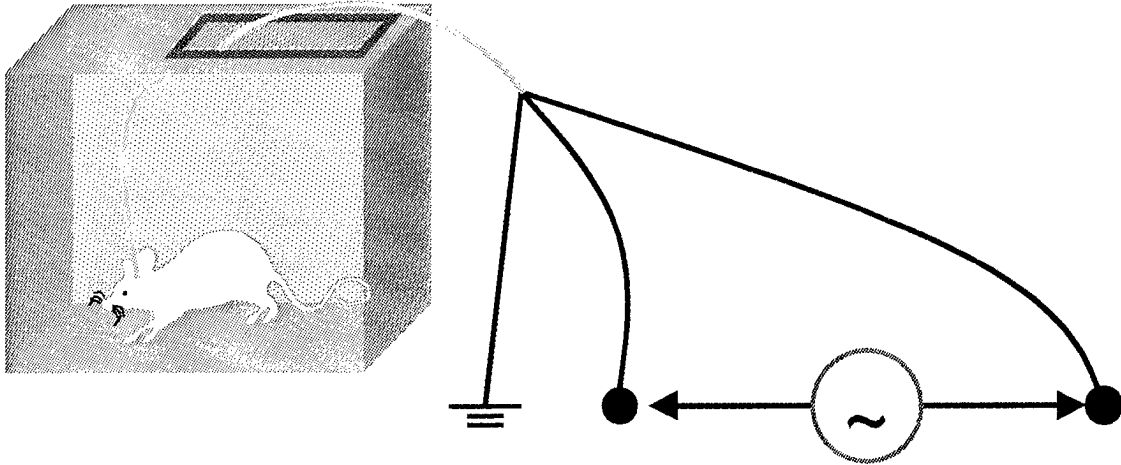# Figure 1

A

Hypothesis
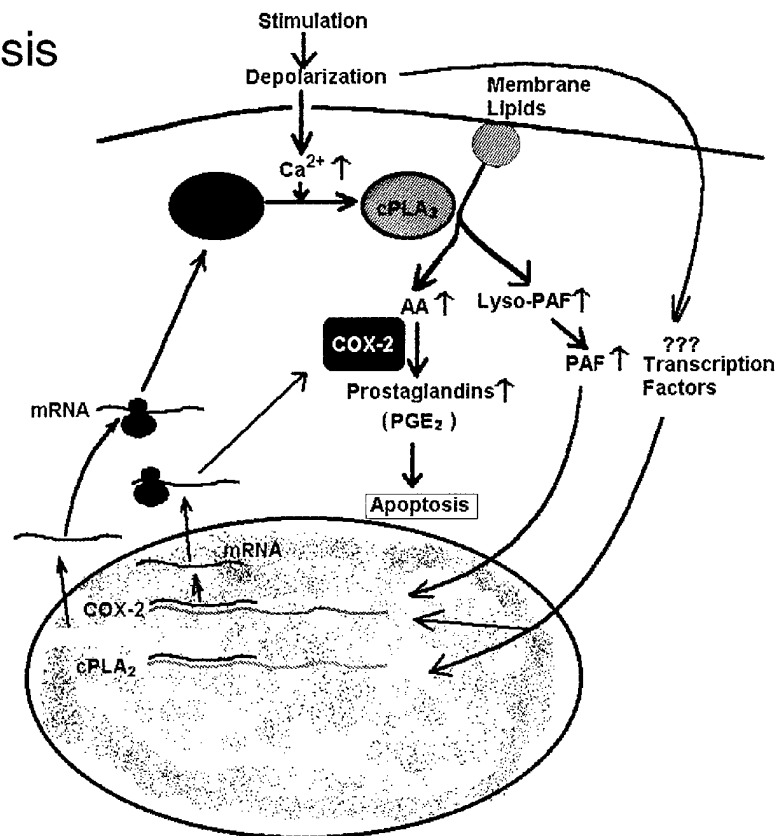
B

Before Kindling

After Kindling

Figure 2

A

Stimulation delivered and after discharge monitored

B
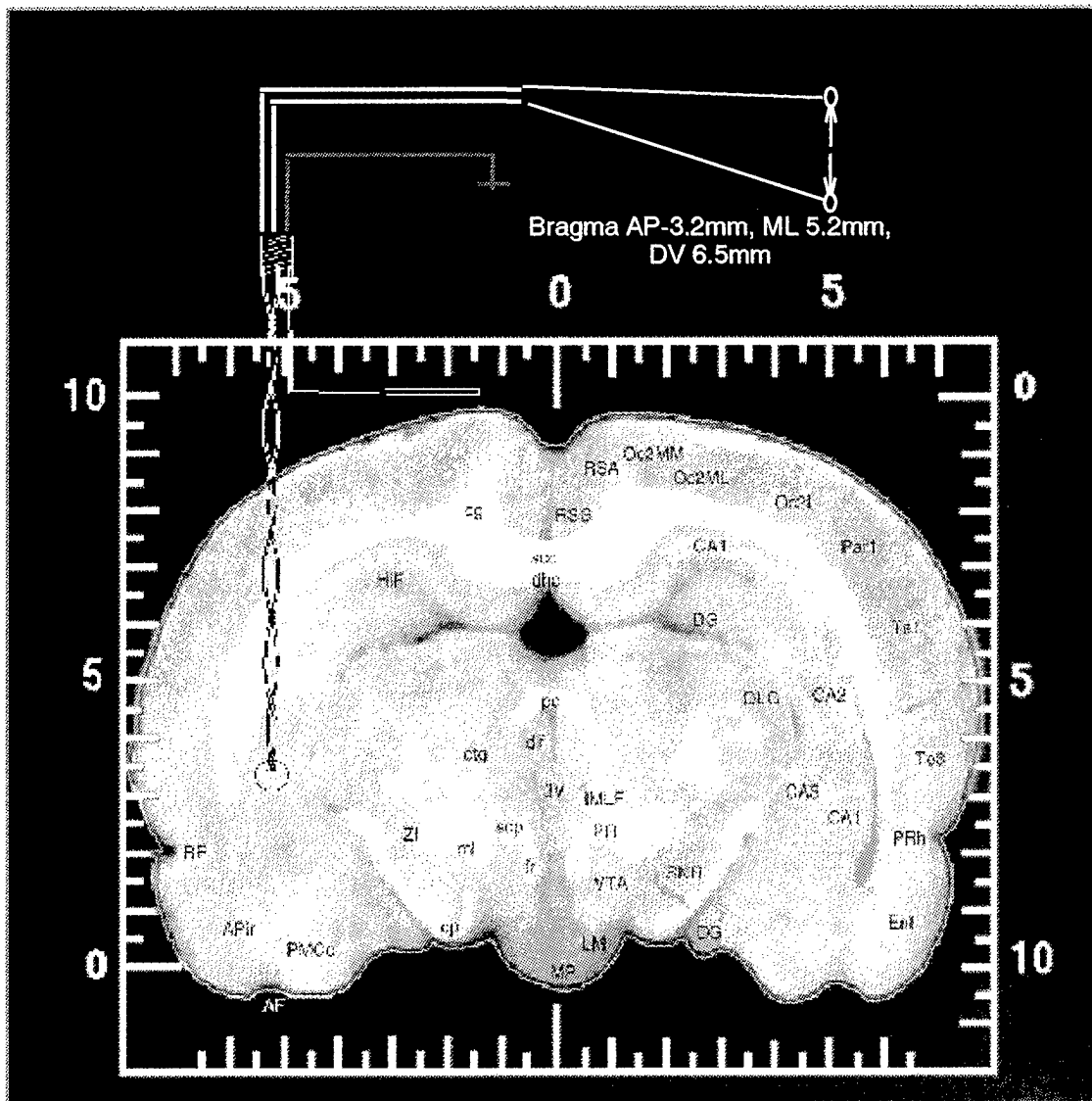

Bragma AP-3.2mm, ML 5.2mm, DV 6.5mm

Figure 3

Kindling Protocol

- 9 days of stimulation every other day.

- 12 daily stimulations every 1/2 hour.

- Each stimulation contains one train of 50 Hz pulses, lasting 10 seconds.

- Each pulse consists of one biphasic square wave pulse, lasting for 2 milliseconds.
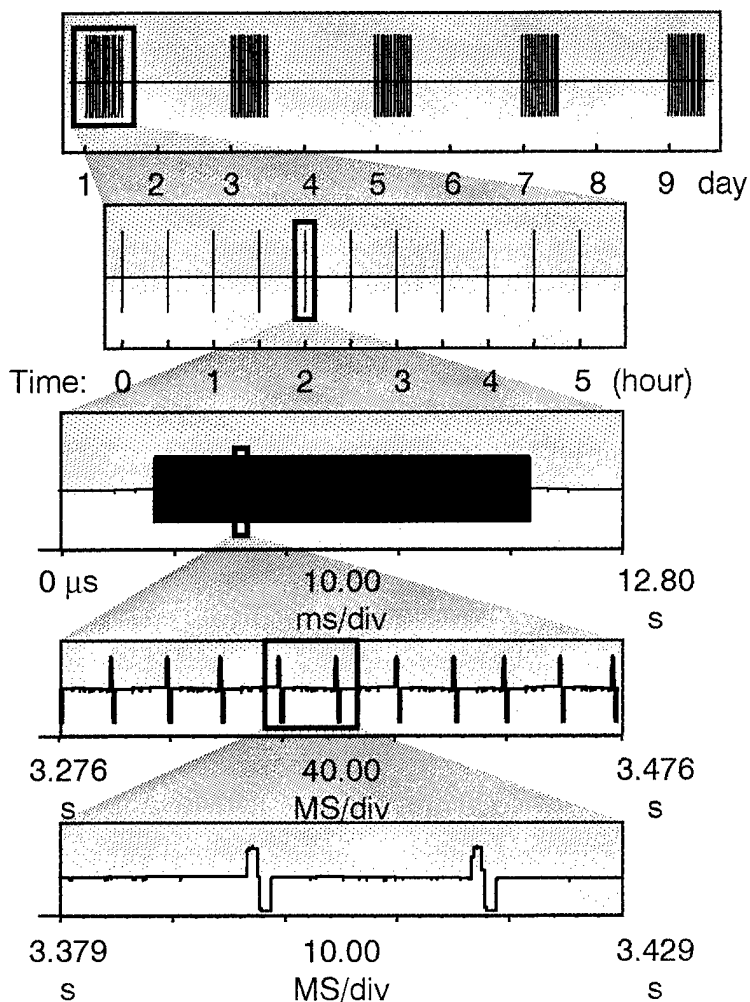
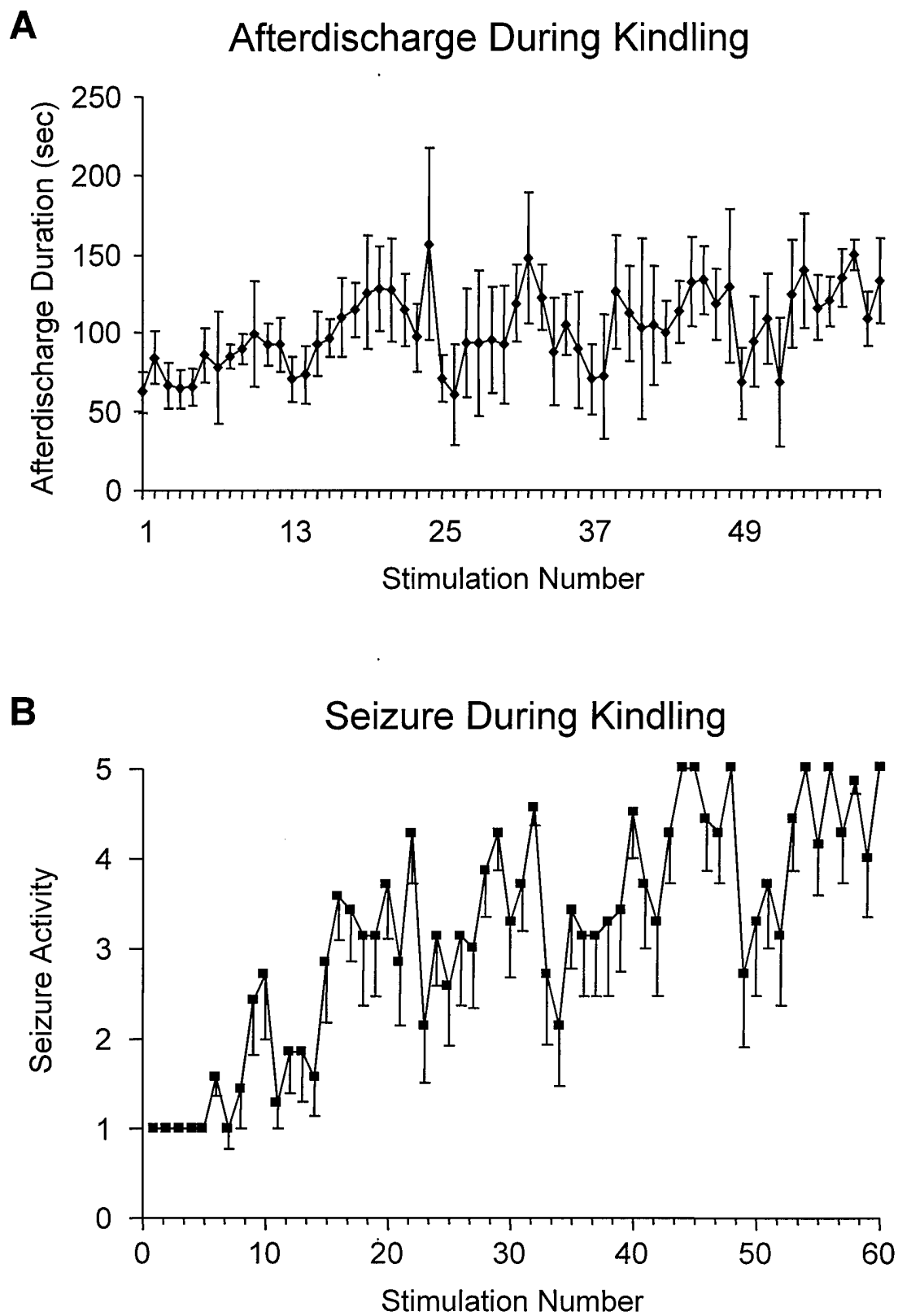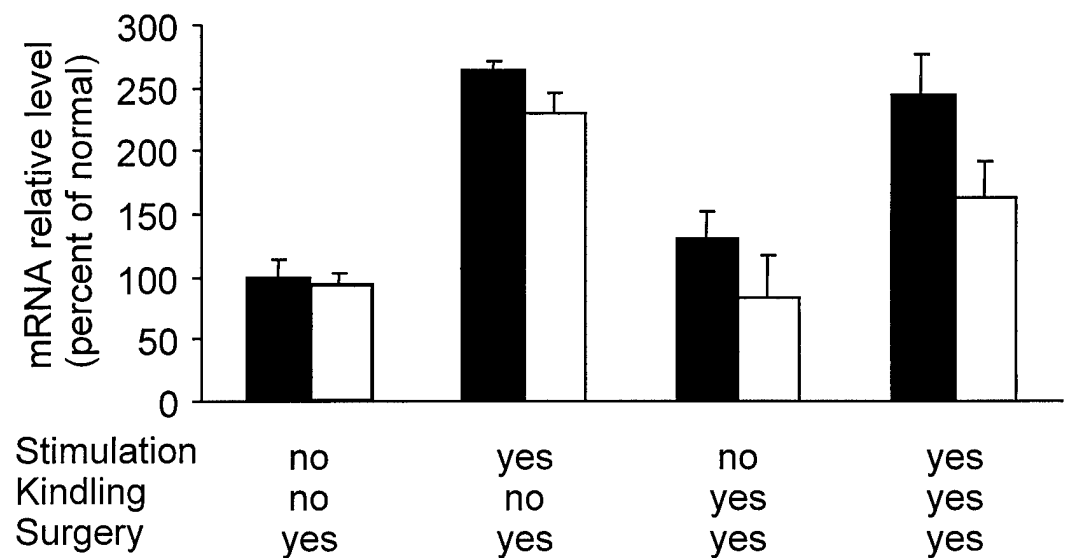- The intensity of stimulation was kept constant at 400µA

Figure 4

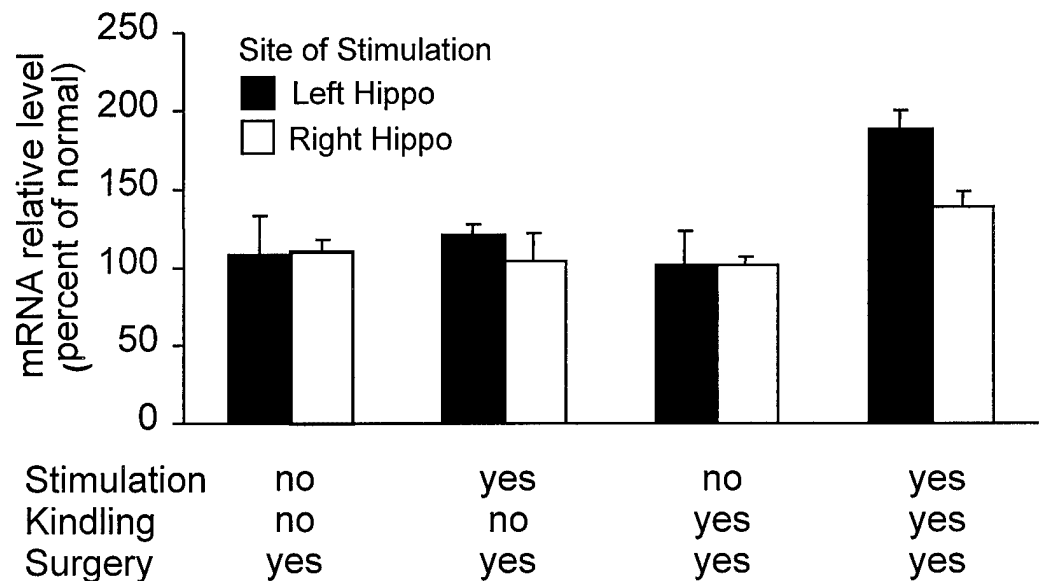**A** Afterdischarge During Kindling

B Seizure During Kindling

Figure 5

A

Stimulation	no	yes	no	yes
Kindling	no	no	yes	yes
Surgery	yes	yes	yes	yes

B

Site of Stimulation
- ■ Left Hippo
- ☐ Right Hippo

Stimulation	no	yes	no	yes
Kindling	no	no	yes	yes
Surgery	yes	yes	yes	yes

Figure 6

A

B

Figure 7

A

Kindled Hippocampus

Naïve Hippocampus

Kindled Cortex

Naïve Cortex

Time after stimulation (hour)

B

Relative mRNA level (Percent control)

Time after stimulation (hour)

- Kindled Left Hippocampus
- Naïve Left Hippocampus
- Kindled Left Cortex
- Naïve Left Cortex

Figure 8

A

Kindled
Hippocampus

Kindled
Cortex

0   1   2   4   6   12   24

Time after stimulation (hour)

B

- ▲ Left Hippocampus
- ○ Left Cortex

Relative mRNA abundance (percent of control)

Time after stimulation (hours)
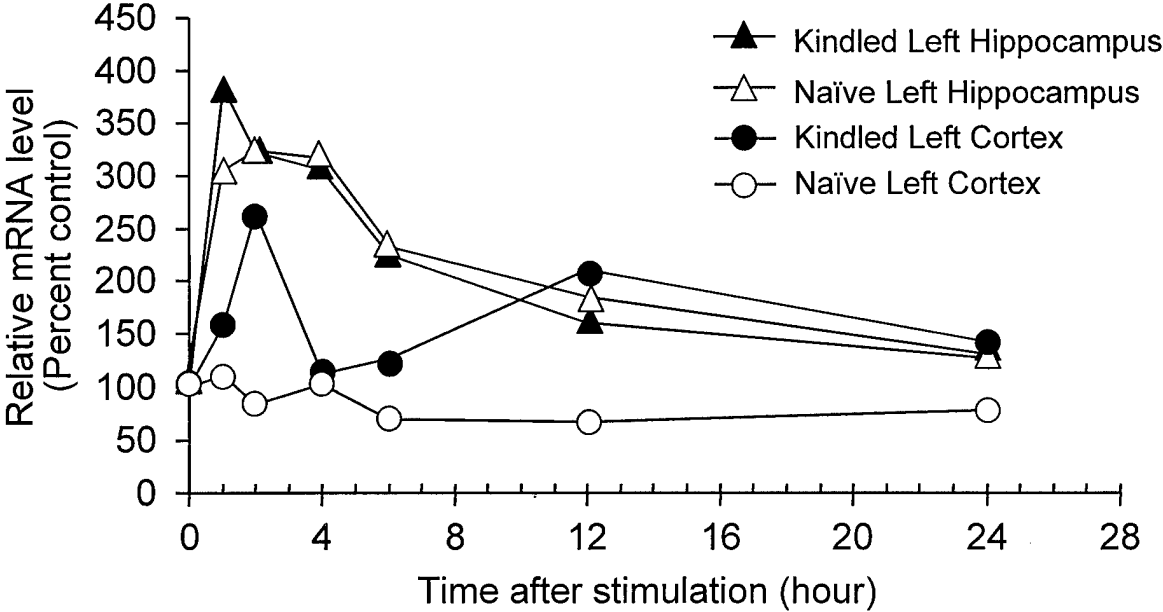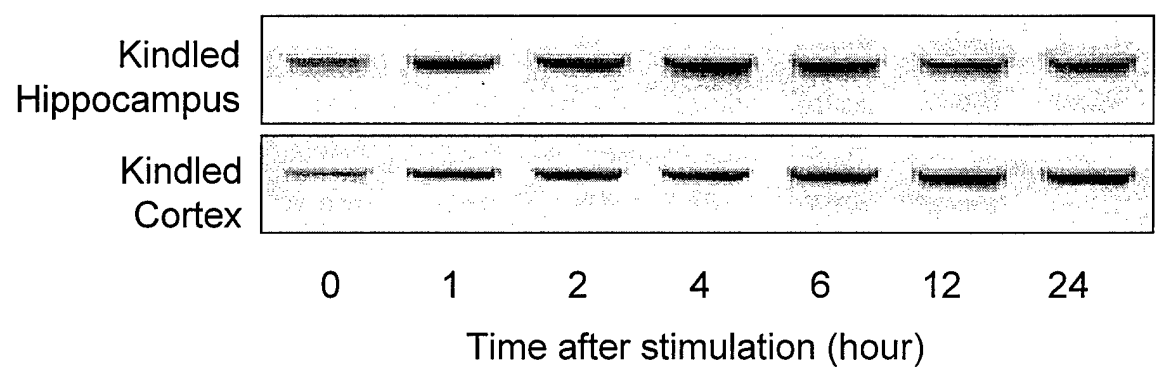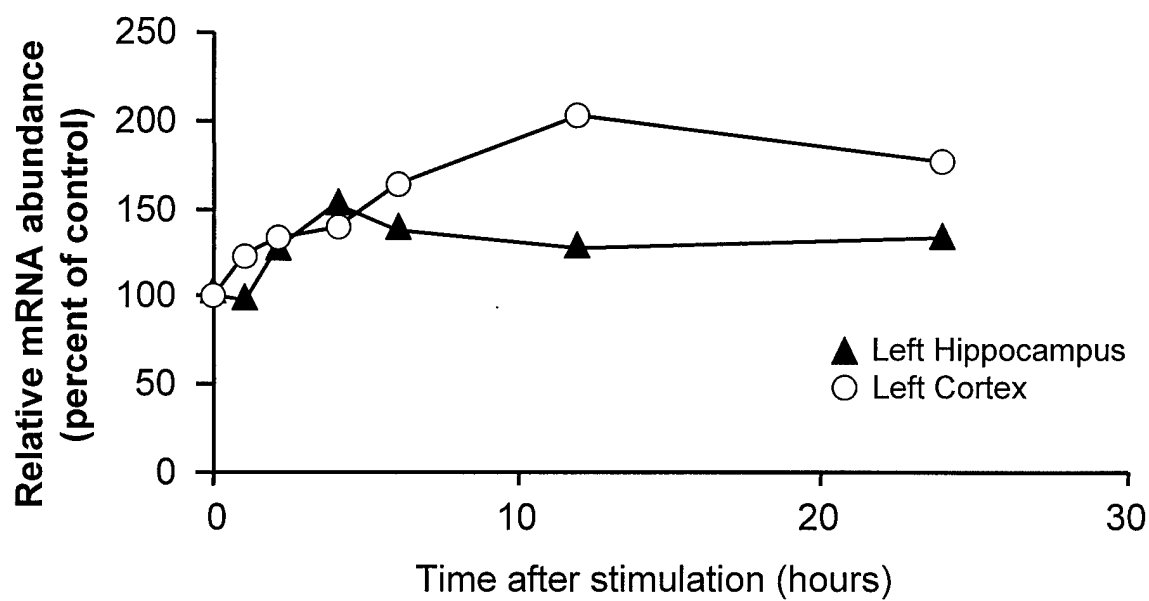
Pretreatment of animals with the intracellular PAF antagonist BN 50730 strongly attenuates COX-2 induction by kainic acid. We are currently testing the hypothesis that the overexpression of the PAF-COX-2 pathway leads to neuronal cell apoptosis in several CNS disease models. Although it is often stated that the inflammatory response is not associated with apoptotic cell death, PAF and COX-2 appear to act as neuron injury messengers without reflecting classical "inflammatory" features to the entire tissue.

Key words: COX-2, PAF, kainic acid, apoptosis, hippocampus, gene expression, neuroprotection

Introduction

Neuronal and glial cell membranes store a wide variety of lipid messengers as part of phospholipid molecules. Receptor-mediated events and changes in intracellular $[Ca^{2+}]$, which occur during excitatory neurotransmission and in activity-dependent synaptic plasticity, activate phospholipases that catalyze the release of bioactive moieties from membrane phospholipid. These messengers then participate in intracellular and/or intercellular signaling pathways. Bioactive lipids have significant neurobiological actions in neurotransmitter release, synaptic plasticity, and programs of neuronal gene expression. Accordingly, much of contemporary research into bioactive lipids has focussed on their neurobiological significance.

Stroke, neurotrauma, epileptic brain damage and, likely, neurodegenerative diseases (e.g., Alzheimer's) disrupt the tightly regulated enzymes that control the production and accumulation of lipid messengers, such as free arachidonic acid, diacylglycerol, and platelet-activating factor, (PAF, 1-0-alkyl-2-acyl-sn-3-phosphocholine), under physiological conditions. Rapid activation of phospholipases, particularly of phospholipase A_2 (PLA_2), occurs at the onset of cerebral ischemia and seizures (Bazan, Rodriguez de Turco, and Allan, 1995). There are a wide variety of PLA_2s (Dennis, 1994), and current investigations aim to define those affected by different pathological conditions. For example, in addition to the role(s) of intracellular PLA_2s in lipid messenger formation, it has recently been discovered that a low molecular weight, secretory PLA_2 synergizes glutamate-induced neuronal damage (Kolko, DeCoster, Rodriguez de Turco, 1996). Therefore, a synaptic secretory PLA_2 may also be a target in these diseases. Whereas pathways leading to PLA_2 activation/release are part of normal neuronal function, ischemia-reperfusion and other pathological conditions enhance these events, overproducing PLA_2-derived lipid messengers involved in neuronal damage (e.g., enzymatically produced arachidonic acid oxygenation

metabolites, non-enzymatically generated lipid peroxidation products, and other reactive oxygen species, PAF). For example, among the consequences of PLA_2 activation by ischemia are alterations in mitochondrial function by the rapid increase in the brain free fatty acid pool size that leads to the uncoupling of oxidative phosphorylation from respiratory chain. The major consequence is the pathological accumulation of lipid messengers.

PAF is a very potent and short-lived lipid messenger. It is known to have a wide range of actions: as a mediator of inflammatory and immune responses, as a second messenger, and as a potent inducer of gene expression in neural systems. Thus, in addition to its acute roles, PAF can potentially mediate longer-term effects on cellular physiology and brain functions. Furthermore, the early response gene, prostaglandin endoperoxide synthase-2 (PGS-2, COX-2, TIS-10), performs a dual function similar to PAF. Prostaglandin G/H synthase-2 catalyzes the cyclooxygenation and peroxidation of arachidonic acid into PGH_2, the precursor of biologically active prostaglandins, thromboxanes, and prostacyclin. PGS-1 also catalyzes the same first committed step of the arachidonic acid cascade. PGS-2, however, is expressed in response to mitogenic and inflammatory stimuli. In contrast, PGS-1 expression is not subject to short-term regulation. Neurons in the hippocampus, as well as in a few other brain regions, are unlike other cells in that they display basal levels of PGS-2 expression (Yamagata *et al.*, 1993). This expression is modulated by synaptic activity, LTP and involves the N-methyl-D-aspartate class of glutamate receptors (Yamagata *et al.*, 1993; Kaufmann *et al.*, 1996). In pathological environments present in seizures, ischemia, and neurodegenerative diseases such as Alzheimer's disease, an increased expression of PGS-2 is observed. Additionally, since PAF is a transcriptional activator of PGS-2 (Bazan *et al.*, 1994), both may be linked as a signaling system in neuronal responses to synaptic activation and in CNS diseases. Moreover, PGS-2 is expressed in neurons (Yamagata *et al.*, 1993), and PAF elicits neuronal plasticity actions (Shimizu and Wolfe, 1990; Clark *et al.*, 1992; Kato *et al.*, 1994). This chapter discusses the significance of PAF and PGS-2 as inflammatory mediators in the CNS.

PAF Modulates Synaptic Plasticity, and in Pathological Conditions, Contributes to Excitotoxicity by Enhancing Glutamate Release

PAF enhances glutamate release in synaptically-paired rat hippocampal neurons in culture (Clark *et al.*, 1992). The PAF analogue methylcarbamyl (mc-PAF), but not biologically inactive lyso PAF, increases excitatory synaptic responses. The inhibitory neurotransmitter γ-aminobutyric acid is unaffected by mc-PAF under these conditions. The presynaptic PAF receptor

antagonist BN-52021 blocks the mc-PAF-enhanced glutamate release. In addition, mc-PAF increases presynaptic glutamate release, since it does not augment the effects of exogenously added glutamate, and it evokes spontaneous synaptic responses characteristic of enhanced neurotransmitter release. Therefore, as a modulator of glutamate release, PAF participates in long-term potentiation (Kato et al., 1994), synaptic plasticity, and memory formation.

Ischemia and seizures increase PAF content in brain (for reference see Bazan, Rodriguez de Turco, and Allan, 1995). Furthermore, brain is endowed with a variety of degradative enzymes that rapidly convert PAF to biologically inactive lyso PAF (Bazan, 1995). Presynaptic membranes display PAF binding that can be displaced by BN-52021, a terpenoid extracted from the leaf of the Ginkgo biloba tree, which binds preferentially to the synaptosomal site (Marcheselli et al., 1990). It is likely that this PAF binding site is the seven transmembrane PAF receptor that has been cloned (Bazan, Rodriguez de Turco, and Allan, 1995). BN-52021 inhibits both PAF-induced glutamate release (Yamagata et al., 1993) and long-term potentiation (Kato et al., 1994). Moreover, this antagonist is neuroprotective in ischemia-reperfusion damage in the gerbil brain (for reference see Bazan, Rodriguez de Turco, and Allan, 1995). Taking these findings together, PAF, when overproduced at the synapse during ischemia, will promote enhanced glutamate release that in turn, through the activation of post-synaptic receptors, will contribute to excitotoxicity.

PAF is a Transcriptional Activator of Prostaglandin Endoperoxide Synthase-2

In addition to its modulatory effect on synaptic transmission and neural plasticity, PAF activates receptor-mediated immediate early gene expression (Squinto et al., 1989; 1990; Bazan et al., 1991). Since PAF is a phospholipid and can pass through membranes, it is rapidly taken up by cells. An intracellular binding site with very high affinity, yet pharmacologically distinct from the presynaptic site, was found in brain (Marcheselli et al., 1990). The synthetic hetrazepine BN-50730 is selective for this intracellular site and blocks PAF-induced gene expression of PGS-2 in transfected cells (Bazan et al., 1994).

PAF is a transcriptional activator of PGS-2, as PAF induces mouse PGS-2 promoter-driven luciferase activity transfected in neuroblastoma cells (NG108-15 or SH-SY5Y) and in NIH 3T3 cells. The intracellular PAF antagonist, BN-50730, inhibits PAF activation of this construct (Bazan et al., 1994). Figure 11.1 outlines the role of PAF as a presynaptic messenger.
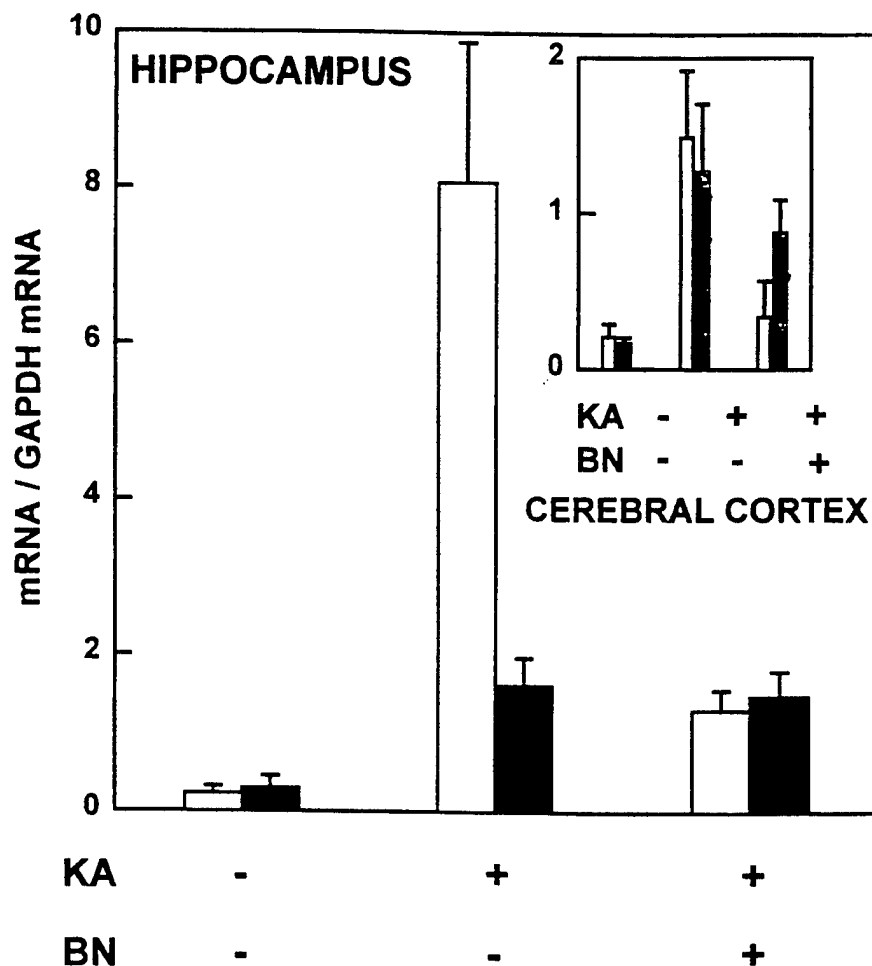
Figure 11.1. Inhibition by BN-50730 of KA-induced PGS-2 mRNA (open bars) and *zif*-268 mRNA (closed bars) accumulation in rat hippocampus and cerebral cortex. Animals were pretreated intracerebroventricularly with BN-50730 in DMSO (experimental) or DMSO alone (control) 15 min before KA injection. RNA was extracted for Northern blot analysis 2 hr after KA injection (n = 6 from two separate experiments, error bars ±1 s.d.). (Published with permission from Marcheselli and Bazan (1996) in *Journal of Biological Chemistry*, 271.)

Sustained Transcriptional Upregulation of PGS-2 Precedes Kainic Acid-Induced Neuronal Damage in Hippocampus

The abundance in brain of several early-response gene transcripts shows rapid and transient increases during cerebral ischemia and after seizures (Morgan and Curran, 1991a; Marcheselli and Bazan). Several early-response genes encode transcription factors that in turn modulate the expression of other genes, whereas others encode inducible enzymes. The glutamate analogue, kainic acid, promotes extensive neuronal damage, particularly in the hippocampus (Pollard *et al.*, 1994), and also induces early-response genes such as the transcription factor *zif*-268 (Marcheselli and Bazan, 1994). PGS-2 is also induced under these conditions, but there are striking

differences in the magnitude and duration of the induction of PGS-2 as compared with *zif*-268. The PGS-2 peak in mRNA abundance was evident at 3 hr (71-fold increase) as compared to 1 hr for *zif*-268 (10-fold increase). *Zif*-268 mRNA time-course of changes in the hippocampus corresponds to the expected profile of early-response genes; i.e., a rapid decrease in abundance after the peak is apparent. PGS-2, on the other hand, displayed sustained upregulation for several hours after kainic acid injection (5.2 fold increase at 12 hr) (Marcheselli and Bazan, 1996). The increased PGS-2 mRNA abundance in hippocampus after kainic acid may be due to enhanced transcription, increased availability of factors that prolong mRNA half-life (e.g., PGS-2 mRNA binding proteins), and/or changes in translational modulation. Therefore, to assess if transcriptional activation is involved in PGS-2 and *zif*-268 induced expression by kainic acid nuclear run-on, transcription assays were conducted. The pattern of transcriptional activation qualitatively matched the profile of changes in mRNA abundance (Figure 11.2B). The level of relative stimulation of PGS-2 transcription was, however, less than the relative increases in mRNA. It should be noted that the methodologies used to obtain these two sets of values differ in several aspects and may not be readily comparable. As a consequence, *in vitro* transcription patterns are to be considered as analogous to mRNA abundance (Marcheselli and Bazan, 1996). Analogous, comparative patterns are also displayed in Figure 11.2A and 11.2B between *in vitro* transcription and mRNA abundance for *zif*-268. The transcriptional activation of PGS-1, as depicted in Figure 11.2B, displayed no induction up to 6 hr; however, there was a small increase after 72 hr of kainic acid treatment.

PGS-2 expression, as analyzed 2 hr after kainic acid injection, was highest in hippocampus (35-fold), followed by cerebral cortex (8-fold) (Figure 11.1). While small increases were observed in brain stem and striatum, there were no changes in cerebellum. The largest induction of *zif*-268 was also observed in hippocampus (5.5-fold) followed by cerebral cortex (4.8-fold). Again, the changes observed in brain stem and striatum were small, with no detectable changes in cerebellum (Figure 11.1). The intracellular PAF receptor antagonist BN-50730 given intracerebro-ventricularly (icv) 15 min prior to kainic acid administration reduces both PGS-2 mRNA (data not shown) and protein (Figure 11.3). Under the same conditions, PGS-1 protein was not affected.

The Platelet Activating Factor-Prostaglandin G/H Synthase-2 Intracellular Signaling Pathway and Apoptosis

A PAF-stimulated signal transduction pathway is a major component of the kainic acid-induced PGS-2 expression in hippocampus. This conclusion is
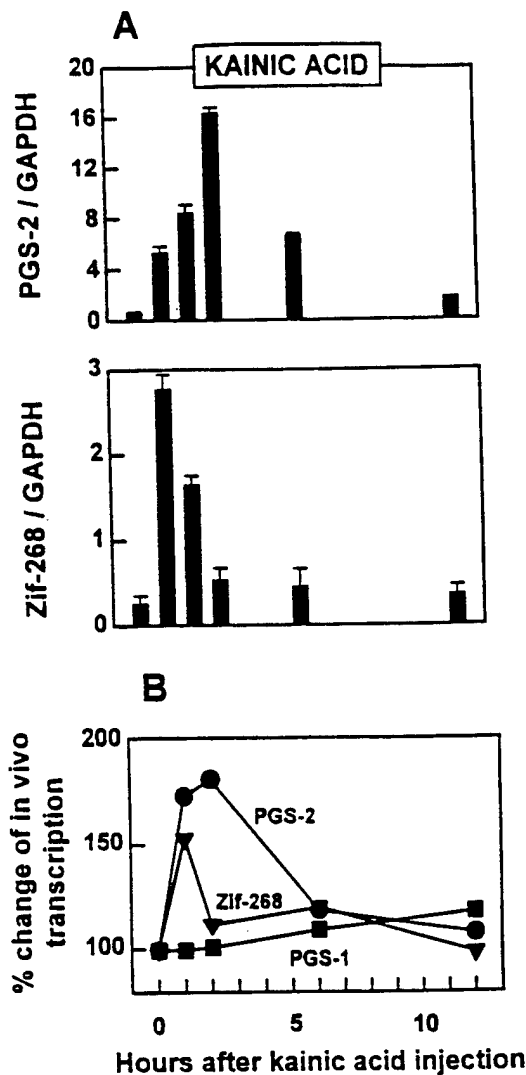
Figure 11.2. Time course of changes in relative mRNA abundance and transcriptional activity in hippocampus after KA treatment or a single electroconvulsive shock (ECS). (A) Relative abundance of PGS-2 and *zif*-268 mRNAs compared to GAPDH mRNA, as assessed by northern analysis (n = 9–12 for each time point from 3 separate experiments, error bars ±1 s.d.) (B) Transcriptional activity of PGS-2, PGS-1, and *zif*-268 genes assessed by nuclear run-on transcription (n = 3–4 from three separate experiments). Data are normalized to transcriptional activity of GAPDH. (Published with permission from Marcheselli and Bazan (1996) in *Journal of Biological Chemistry*, 271.)

based upon the finding that (a) PAF induces mouse PGS-2 promoter-driven luciferase activity in transfected cells, and BN-50730 inhibits this effect (Bazan *et al.*, 1994); and (b) BN-50730 inhibits kainic acid-induced PGS-2 mRNA and protein accumulation in hippocampus by 90% (Bazan *et al.*, 1994). Both PAF (Prescott, Zimmerman, and McIntyre, 1990) and PGS-2 (Bazan, Botting, and Vane, 1996) are potent mediators of the injury/inflammatory response (Clark *et al.*, 1992; Kato *et al.*, 1994). PAF and PGS-2 (Yamagata *et al.*, 1993; Kaufmann *et al.*, 1996) are also interrelated in neuronal plasticity. The PAF transcriptional activation of PGS-2 may provide clues about novel neuronal cell death pathways. The antagonist
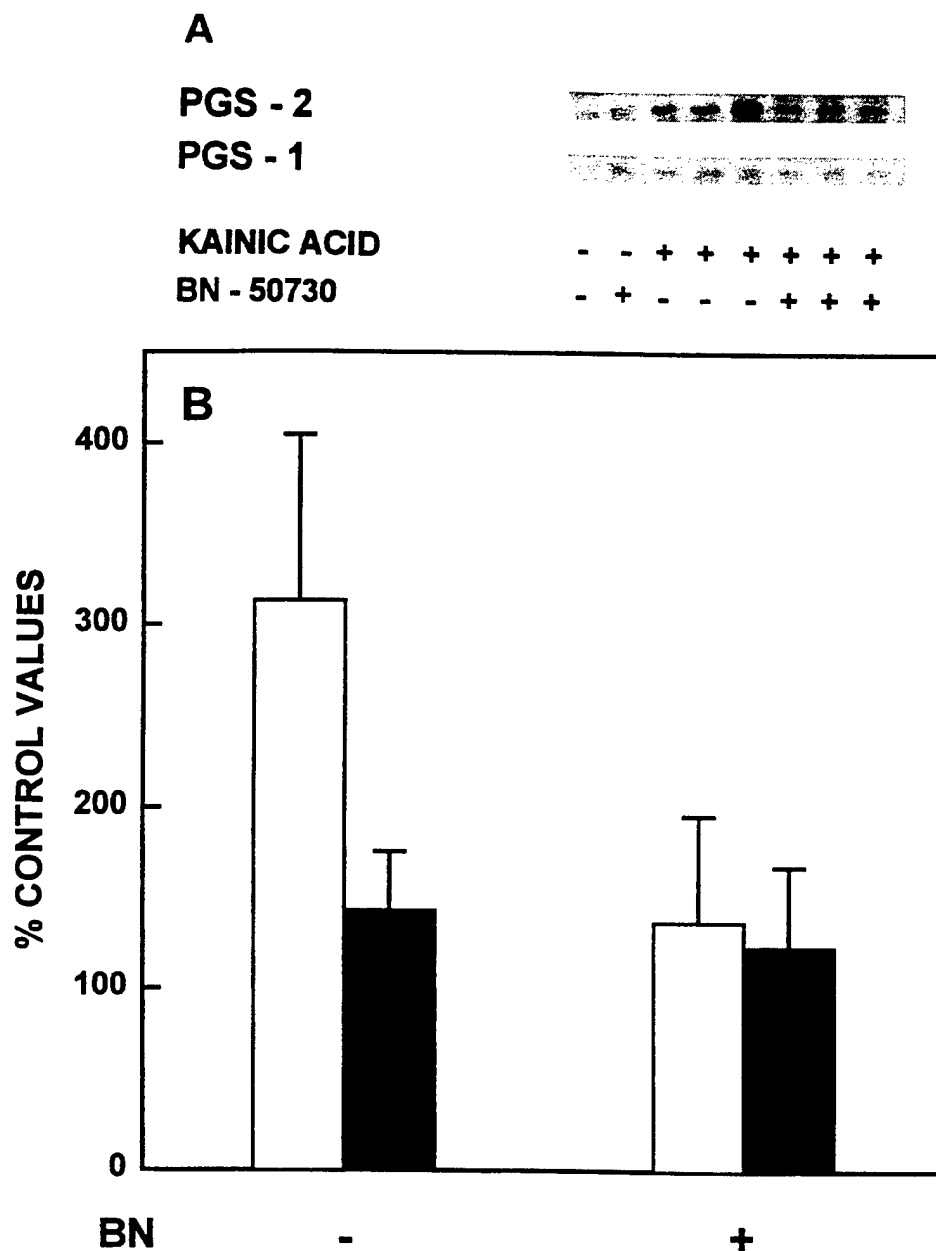
Figure 11.3. Inhibition by BN-50730 pretreatment of KA-induced PGS-2 protein accumulation (open bars), but not of endogenous PGS-1 (closed bars) in rat hippocampus. (A) Representative Western blots. BN-50730 or vehicle treatments were as for Figure 11.4. Samples were collected 6 hr after KA treatment. (B) Quantification of Western blot data expressed as a percent increase over control (vehicle-pretreated) values (n = 10–12 from three separate experiments, error bars ±1 s.d.). (Published with permission from Marschelli and Bazan (1996) in *Journal of Biological Chemistry*, **271**.)

BN-50730 was much less effective against *zif*-268 expression (Figure 11.4). In fact, the delayed hippocampal induction of PGS-2 by kainic acid precedes selective neuronal apoptosis by this agonist in this neuroanatomical region (Pollard *et al.*, 1994).
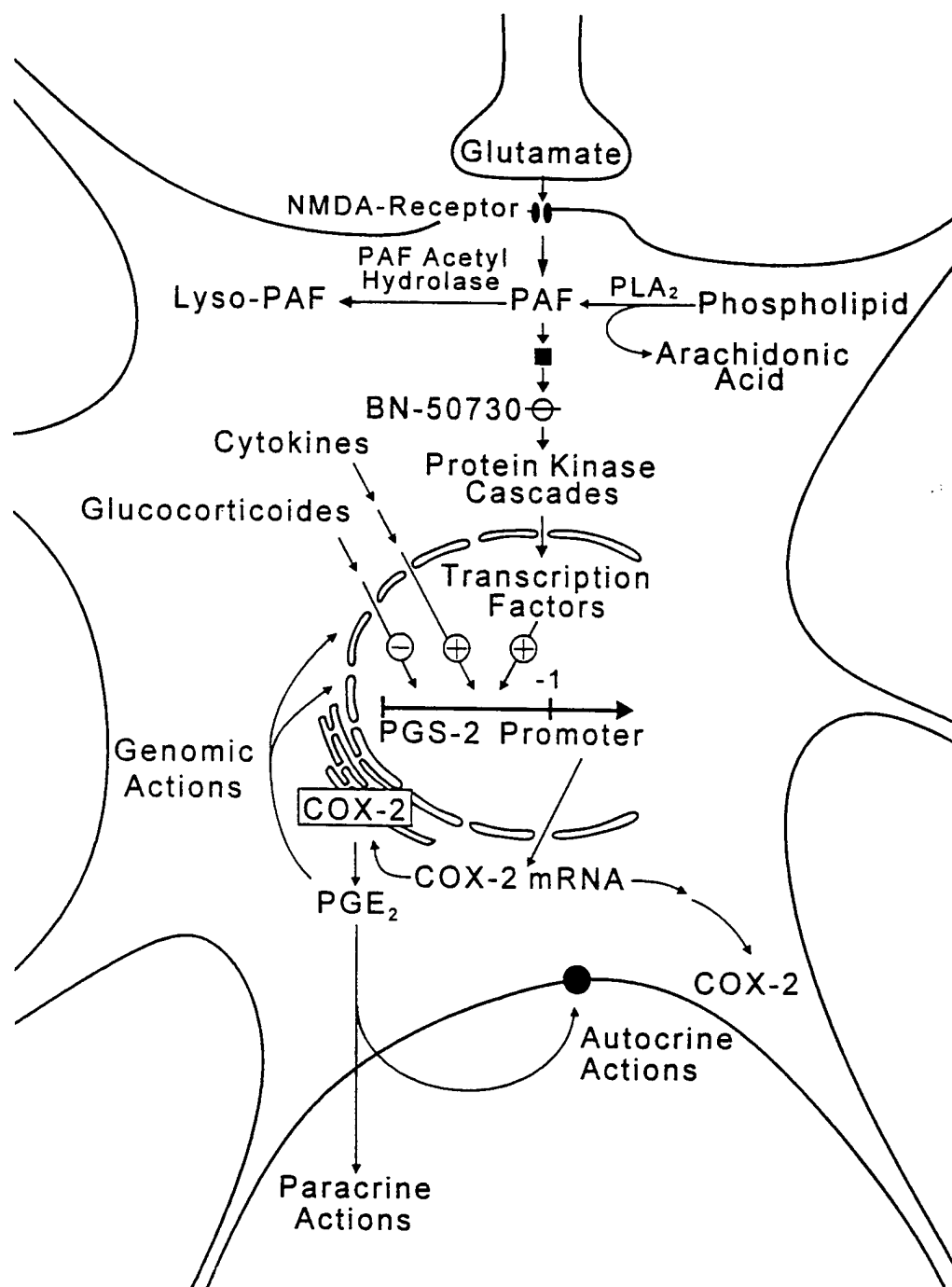
Figure 11.4. Excitatory synaptic transmission enhances the production of PAF, other messengers, and the expression of PGS-2. NMDA-receptor activation by glutamate leads to phospholipase A_2 activation and the generation of PAF and arachidonic acid. PAF is synthesized through other metabolic routes as well (10). Other synaptic events, in addition to NMDA-receptor, may also be involved in the accumulation of PAF. PAF activates PGS-2 gene expression through a BN-50730-sensitive intracellular site, protein kinase cascades, and transcription factors. The PGS-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). PGS-2 protein (COX-2) then catalyzes the conversion of arachidonic acid into PGH_2, the precursor of eicosanoids. Constitutive PGS-1 also catalyzes this metabolic step (not shown). Prostaglandin E_2 is depicted as a product of PGS-2 (COX-2). The specific products of overexpressed PGS-2 are not yet known. PGE_2 is shown to potentially elicit genomic, paracrine, and/or autocrine effects.

In neurotrauma, cerebrovascular and neurodegenerative diseases, the significance of the PLA_2-related signaling triggered by ischemia reperfusion may be part of events that are finely balanced between neuroprotection and neuronal cell death. The precise events that would tilt this balance toward the latter are currently being investigated. We are exploring the hypothesis that PAF-COX-2 is a common signaling pathway involved in neurodegenerative diseases as diverse as retinitis pigmentosa and Alzheimer's Disease. It is interesting to note that PAF, being short-lived and rapidly degraded by PAF acetylhydrolase (Bazan, 1995; Tjoelker *et al.*, 1995), behaves as a long-term signal with consequences to neurons through PGS-2 sustained expression. It is also interesting that the PAF-PGS-2 pathway may be activated in neurons as an early event in the pathophysiology of several diseases, and although this pathway is a component of the "classical inflammatory" response, it is restricted to a disruption of intracellular signaling. In fact, apoptosis, by definition, lacks an initial inflammatory component. PGS-2 is localized in the nuclear envelope and perinuclear endoplasmic reticulum. The overexpression of hippocampal PGS-2 during cerebral ischemia and seizures may lead, in turn, to the formation of neurotoxic metabolites (e.g., superoxide). Current investigations are attempting to determine whether other messengers cooperate to enhance neuronal damage (e.g., nitric oxide), as well as examining the possible involvement of astrocytes and microglial cells. Further understanding of these potentially neurotoxic events involving lipid messengers and PGS-2 will permit the identification of new strategies and define therapeutic windows for the management of the inflammatory component in stroke, epileptic brain damage, head injury, and neurodegenerative diseases.

Acknowledgments

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Second Messengers, Long-Term Potentiation, Gene Expression and Epileptogenesis

Nicolas G. Bazan and Michael J. Serou

Louisiana State University Medical Center,
School of Medicine, Neuroscience Center of Excellence
New Orleans, Louisiana

Address correspondence to: Nicolas G. Bazan, Neuroscience Center of Excellence, Louisiana State University Medical Center School of Medicine, 2020 Gravier Street, Suite B, New Orleans, Louisiana 70112

Phone: 504-599-0832
FAX: 504-568-5801
E-mail: nbazan@lsumc.edu

Running title: Second messengers, LTP, genes and epileptogenesis

Introduction

Various forms of brain damage triggers cellular and molecular events that often result in impaired synaptic plasticity that, in turn, induces recurrent seizures and epileptic discharges in the electroencephalogram. Hippocampal sclerosis, frequently the result of early neurotrauma or other forms of brain damage, is thought to be a significant event in epileptogenesis. Our hypothesis is that epileptogenesis involves a synaptic signaling dysfunction engaging lipid messengers, some of which modulate cell function. Synaptic messengers participate in synaptic strengthening, known also as long-term potentiation (LTP), a cellular form of memory formation. The persistence of changes in LTP indicates that synaptic plasticity has taken place and suggests that signal transduction includes modulation of gene expression. In fact, LTP induces several early response genes, including transcription factors, which, in turn, regulate the expression of gene cascades as well as of genes that encode proteins which participate in other functions such as extracellular matrix development and remodeling. Therefore perturbing synaptic signaling may contribute to aberrant synaptic plasticity and epileptogenesis. Once that abnormal connections are established, enhanced excitability promotes and contributes to seizure development and propagation. As a consequence, further seizures trigger additional damage. This vicious cycle of events in epileptogenesis has received experimental support (e.g., 1-6).

Phospholipases A_2, Arachidonic Acid and PAF

Phospholipid molecules of membranes from neurons and glial cells store a wide variety of lipid messengers. Receptor-mediated events and changes in intracellular $[Ca^{2+}]$, as occurs during excitatory neurotransmission and in activity-dependent synaptic plasticity, modulate phospholipases that catalyze the release of bioactive moieties from phospholipids. These lipid messengers then

participate in intracellular and/or intercellular signaling. Bioactive lipids have significant neurobiological actions such as in the function of ion channels, receptors, neurotransmitter release, synaptic plasticity, and programs of neuronal gene expression. As a result, there is a rapid increase in the brain free fatty acid pool size, particularly of arachidonic acid (7, 8) and of the bioactive phospholipid, platelet-activating factor, (PAF, 1-0-alkyl-2-acetyl-sn-3-phosphocholine)(9, 10). Arachidonic acid is converted into a variety of biologically active derivatives through cyclooxygenation (11) and lipoxygenation (12), with these conversions being particularly enhanced during reperfusion or repeated seizures, when tissue oxygenation is coupled with an abnormally large free arachidonic acid pool. These early findings, as well as more recent ones (13) have suggested the involvement of the cytosolic, calcium dependent phospholipase A_2 (cPLA$_2$) in brain damage. This enzyme, which has a preference for arachidonoyl phospholipids, catalyzes the release from membranes of free arachidonic acid and of the PAF precursor, lyso PAF. The importance of cPLA$_2$ in brain injury is strongly supported by the recent finding that cPLA$_2$ knockout mice have substantially reduced infarcts and lesser neurological deficits following transient middle cerebral artery occlusion (14). While these observations do not demonstrate that cPLA$_2$ is directly involved in epileptogenesis, several ongoing studies are addressing this question.

Brain damage and seizures disrupt the tightly regulated events that control the production and accumulation of lipid messengers, such as free arachidonic acid, diacylglycerol and PAF under physiological conditions. Rapid activation of phospholipases, specifically of phospholipase A_2 (PLA$_2$) as it occurs at the onset of injury and seizures (7, 8), was predicted to play a central role in excitable membrane function and pathology (7). There are a wide variety of PLA$_2$s (15), and current investigations aim to define those involved in synaptic events. In addition to the role(s) of

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intracellular PLA_2s in lipid messenger formation, it has recently been discovered that a low molecular weight, secretory PLA_2 synergizes glutamate-induced neuronal damage (16). Whereas pathways leading to PLA_2 activation/release are part of normal neuronal function, seizures and injury enhance these events, overproducing PLA_2-derived lipid messengers, (e.g. enzymatically produced arachidonic acid oxygenation metabolites and non-enzymatically generated lipid peroxidation products), involved in neuronal damage. Among the consequences of PLA_2 activation by seizures are alterations in mitochondrial function by the rapid increase in the brain free fatty acid pool size (e.g. uncoupling of oxidative phosphorylation from respiratory chain) (17, 18), the generation of lipid messengers and also by impairments in mitochondrial function that, in turn, lead to enhanced release of reactive oxygen species to the cytoplasm.

PAF is a very potent and short-lived lipid messenger. It is known to have a wide range of actions: as a mediator of inflammatory and immune responses, as a second messenger, and as an inducer of gene expression. Thus, in addition to its acute roles, PAF can potentially participate in long-term modifications of cellular physiology and brain function. In this article, the significance of the messenger PAF in synaptic function, including LTP, and neuronal gene expression relevant to epileptogenesis is discussed.

PAF enhances excitatory synaptic transmission and modulates synaptic plasticity.

PAF enhances glutamate release in synaptically-paired rat hippocampal neurons in culture (19). The PAF analog methylcarbamyl (mc-PAF), but not biologically inactive lyso PAF, increases excitatory synaptic responses. The inhibitory neurotransmitter γ-aminobutyric acid is unaffected by mc-PAF under these conditions. The presynaptic PAF receptor antagonist BN 52021 (see below)

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blocks the mc-PAF-enhanced glutamate release. In addition, mc-PAF increases presynaptic glutamate release, since it does not augment the effects of exogenously added glutamate, and it evokes spontaneous synaptic responses characteristic of enhanced neurotransmitter release. Therefore, as a modulator of glutamate release, PAF participates in long-term potentiation (20, 21), synaptic plasticity and memory formation.

PAF, a neuronal injury messenger, contributes to excitotoxicity by enhancing glutamate release.

Seizures and ischemia increase PAF content in brain (9, 10). Furthermore, brain is endowed with a variety of degradative enzymes that rapidly convert PAF to biologically inactive lyso PAF (22-24). Presynaptic membranes display PAF binding that can be displaced by BN 52021, a terpenoid extracted from the leaf of the *Ginkgo biloba* tree, which binds preferentially to the synaptosomal site (25). It is likely that this PAF binding site is the seven transmembrane PAF receptor (26-28). BN 52021 inhibits both PAF-induced glutamate release (19) and long-term potentiation (20-21). Moreover, this antagonist is neuroprotective in ischemia-reperfusion damage in the gerbil brain (29). Taking these finding together, PAF, when overproduced at the synapse during seizures, will promote enhanced glutamate release that in turn, through the activation of post-synaptic receptors, will contribute to enhanced excitotoxicity.

PAF is a transcriptional activator of the early response gene, prostaglandin endoperoxide synthase-2 (COX-2).

In addition to its modulatory effect on synaptic transmission and neural plasticity, PAF activates receptor-mediated immediate early gene expression (30-33). Since PAF is a phospholipid

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and can pass through membranes, it is rapidly taken up by cells. An intracellular binding site with very high affinity, yet pharmacologically distinct from the presynaptic site, was found in brain (25). The synthetic hetrazepine BN 50730 is selective for this intracellular site and blocks PAF-induced gene expression of the inducible form of prostaglandin G synthase in transfected cells (34).

Prostaglandin G/H synthase-2 (PGS-2, COX-2, TIS-10) catalyzes the cyclooxygenation and peroxidation of arachidonic acid into PGH_2, the precursor of biologically active prostaglandins, thromboxanes and prostacyclin (35-36). COX-1 also catalyzes the same first committed step of the arachidonic acid cascade. COX-2, however, is expressed in response to mitogenic and inflammatory stimuli and is encoded by an early-response gene. In contrast, COX-1 expression is not subject to short-term regulation. Neurons in the hippocampus, as well as in a few other brain regions, are unlike other cells in that they display basal levels of COX-2 expression (37). This expression is modulated by synaptic activity, LTP and involves the N-methyl-D-aspartate class of glutamate receptors (37, 38).

PAF is a transcriptional activator of COX-2, as PAF induces mouse COX-2 promoter-driven luciferase activity transfected in neuroblastoma cells (NG108-15 or SH-SY5Y) and in NIH 3T3 cells. The intracellular PAF antagonist, BN 50730, inhibits PAF activation of this construct (34). Figure 1 outlines the role of PAF as a presynaptic messenger.

Sustained transcriptional upregulation of COX-2 during kainic acid-induced seizures in hippocampus.

The abundance in brain of several early-response gene transcripts shows rapid and transient increases during cerebral ischemia and after seizures (32). Several early-response genes encode

transcription factors which in turn modulate the expression of other genes, whereas others encode inducible enzymes. The glutamate analog, kainic acid, when systemically injected in rats, promotes extensive neuronal damage, and a status epilepticus-like condition (particularly in the hippocampus). It also induces early-response genes such as the transcription factor *zif*-268. COX-2 is also induced under these conditions, but there are striking differences in the magnitude and duration of the induction of COX-2 as compared with *zif*-268 (Fig 2). Kainic acid-induced upregulation of COX-2 and *zif*-268 transcription in hippocampus differed , as did the time course of expression following a single seizure triggered by electroconvulsive shock (39). In both conditions, the peak of COX-2 mRNA abundance was later than *zif*-268. In kainic acid treated animals, COX-2 reached a 71-fold increase over controls in 3 hours, whereas *zif*-268 peaked at 1 hr with a 10-fold increase. COX-2 mRNA, 2 hr after kainic acid injection, showed a 35-fold increase in hippocampus as compared with only a 5.5 fold increase in *zif*-268 (39). Also COX-2 peak in mRNA abundance was evident at 3 hrs (71-fold increase) as compared with 1 hr for *zif*-268 (10-fold increase). *Zif*-268 mRNA time-course of changes in the hippocampus corresponds to the expected profile of early-response genes, i.e., a rapid decrease in abundance after the peak. COX-2 on the other hand, displayed sustained upregulation for several hours after kainic acid injection (5.2 fold increase at 12 hours) (39).

Possible significance of the platelet activating factor-COX-2 intracellular signaling pathway in epileptogenesis.

A PAF-stimulated signal transduction pathway is a major component of the kainic acid-induced COX-2 expression in hippocampus. This conclusion is based upon the finding that a) PAF induces mouse COX-2 promoter-driven luciferase activity in transfected cells, and BN 50730

inhibits this effect (34); and b) BN 50730 (given intracerebroventricularly 15 min prior to kainic acid) inhibits kainic acid-induced COX-2 mRNA accumulation in hippocampus by 90% (39). Both PAF (23) and COX-2 (40) are potent mediators of the injury/inflammatory response. PAF (19-21) and COX-2 (37, 38) are also interrelated in neuronal plasticity. The PAF transcriptional activation of COX-2 may provide clues about novel neuronal cell death pathways. The antagonist BN 50730 was much less effective against *zif*-268 expression. In fact, the delayed hippocampal induction of COX-2 by kainic acid precedes selective neuronal apoptosis by this agonist in this neuroanatomical region (41).

In epilepsy, the significance of the PLA_2-related signaling triggered by seizures or injury may be part of events finely balanced between neuroprotection and neuronal cell death. The precise events that would tilt this balance toward the latter are currently being explored. It is interesting to note that PAF, being short-lived and rapidly degraded by PAF acetylhydrolase (42), is a long-term signal with consequences to neurons though COX-2 sustained expression. COX-2 is localized in the nuclear envelope and perinuclear endoplasmic reticulum. The overexpression of hippocampal COX-2 during cerebral ischemia and seizures may in turn lead to the formation of neurotoxic metabolites (e.g. superoxide). Current investigations aim to determine whether or not other messengers cooperate to enhance neuronal damage (e.g. nitric oxide) and the possible involvement of astrocytes and microglial cells. Further understanding of these potentially neurotoxic events involving lipid messengers and COX-2 will permit the identification of new therapeutic strategies for the management of epilepsy.

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Figure 1: **Seizure-triggered signaling events linking synapse activation and COX-2 gene expression in neurons**. NMDA-receptor activation by glutamate leads to phospholipase A_2 activation and the generation of PAF and of arachidonic acid. PAF is synthesized through other metabolic routes as well (19). PAF activates COX-2 gene expression through a BN-50730-sensitive intracellular site, protein kinase cascades and transcription factors. The COX-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). COX-2 protein then catalyzes the conversion of arachidonic acid into PGH_2, the precursor of eicosanoids. Constitutive COX-1 also catalyzes this metabolic step (modified from N. Bazan, *Primer Cerebrovascular Diseases*. M Welsh, L Chaplan, D Reis, B Siesjö, B Weir (eds), Academic Press, 1997, with permission).

Figure 2. **COX-2 and *zif*-268 during kainic acid-induced seizures: Time course of changes in relative mRNA abundance and transcriptional activity in hippocampus.**

(A) Relative abundance of COX-2 and *zif*-268 mRNAs compared to GAPDH mRNA, as assessed by northern analysis. (n=9-12 for each time point from 3 separate experiments, error bars ± 1 s.d.).

(B) Transcriptional activity of COX-2, COX-1 and *zif*-268 genes assessed by nuclear run-on transcription. (n=3-4 from 3 separate experiments). Data are normalized to transcriptional activity of GAPDH (modified from Marcheselli and Bazan, *J. Biol. Chem*, 1996, with permission).
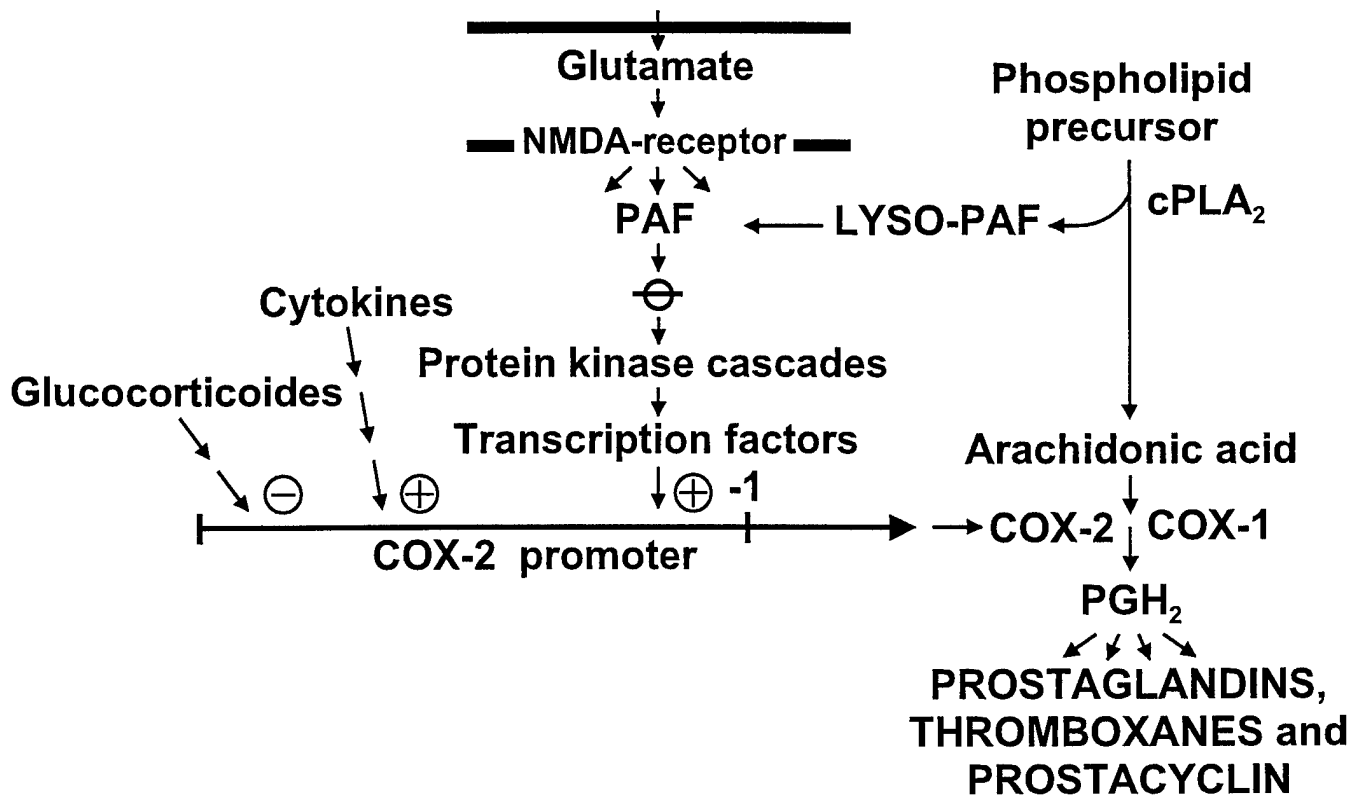
Figure 3. **Inhibition by intracerebroventricular injection of kainic acid-induced COX-2 protein accumulation (open bars), but not of COX-1 (closed bars) in rat hippocampus.**
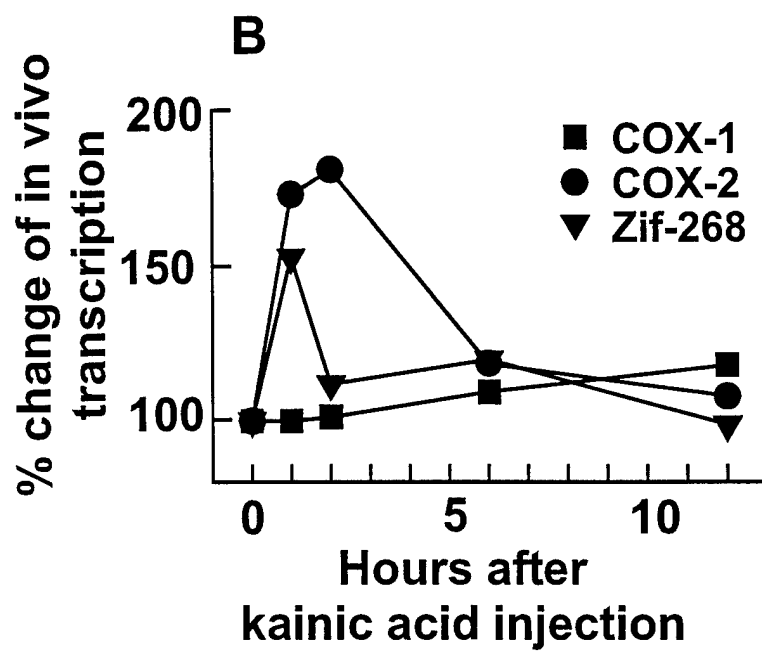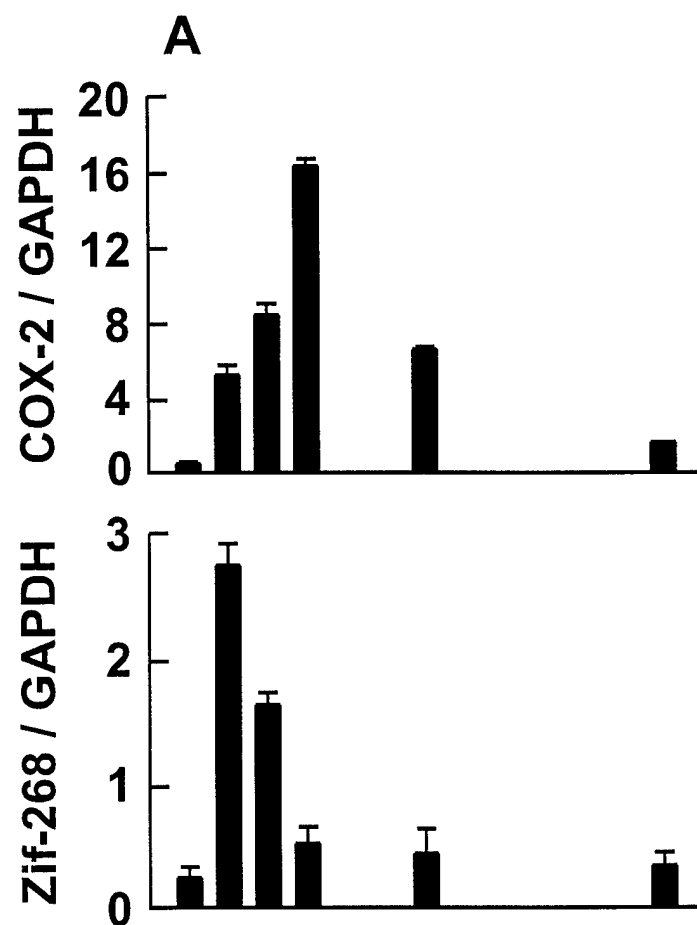
(A) Representative Western blots. Animals were pretreated intracerebroventricularly with BN-50730 in DMSO (experimental) or DMSO alone (control) 15 min before KA injection. Samples were
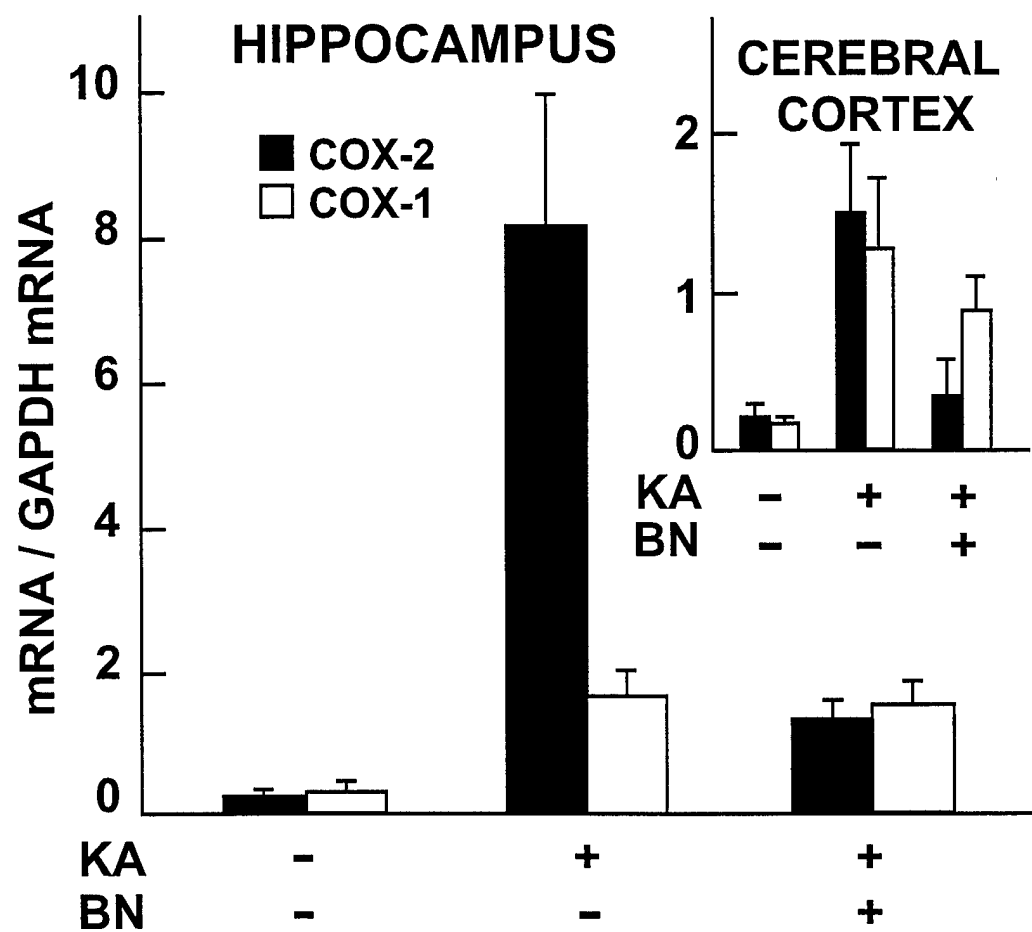
15

collected 6 hours after KA treatment.

(B) Quantification of Western blot data expressed as a percent increase over control (vehicle-pretreated) values. (n=10-12 from 3 separate experiments, error bars ± 1 s. d.) (from Marcheselli and Bazan, *J. Biol. Chem*, 1996, with permission)

Figure 4.   **Potential sites of drug action on the PAF - COX-2 axis.** Excitatory synaptic transmission enhances the production of PAF and of other messengers. PAF activates transcription of COX-2. COX-2 protein is located in the perinuclear region. It is not known the nature of the eicosanoids generated. PGE_2 is depicted as a product that in turn may elicit on the nuclear, paracrine and autocrine actions. Three possible drug targets are indicated as 1, 2 or 3.

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Glutamate

NMDA-receptor

Phospholipid
precursor

PAF ← LYSO-PAF ← cPLA$_2$

Cytokines

Glucocorticoides

⊖

Protein kinase cascades

Transcription factors

Arachidonic acid

⊖ ⊕ ⊕ -1

COX-2 promoter → → COX-2 | COX-1

PGH$_2$

PROSTAGLANDINS,
THROMBOXANES and
PROSTACYCLIN

Stimulus

③ Glucocorticoids

① PAF acetyl hydrolase

Mediator/s (PAF)

PAF-R

② Selective PAF antagonists

Glc-R

Transcription factor

COX-2 gene

COX-2 mRNA

COX-2

PGs ◄— AA

④ Specific COX-2 inhibitors

Effects

⑤ Prostanoid receptor agonists/ antagonists